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EFFECTS OF ADENOSINE ON ETHANOL-INDUCED MODIFICATIONS OF LIVER METABOLISM

ROLE OF HEPATIC REDOX STATE, PURINE AND FATTY ACID METABOLISM

J. ADOLFO GARCÍA-SÁINZ, ROLANDO HERNÁNDEZ-MUÑOZ, WALTER GLENDER, ENRIQUE PIÑA and VICTORIA CHAGOYA DE SÁNCHEZ

Departmento de Bioenergética, Centro de Investigaciones en Fisiología Celular and Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, México 20, D.F., México

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Abstract—A total prevention of ethanol-induced fatty liver by the simultaneous administration of adenosine and allopurinol was observed. Under these conditions, adenosine ameliorated the reduction in the cytoplasmic NAD^+/NADH ratio produced by ethanol metabolism, increased the rate of ethanol oxidation, and decreased the blood ketone bodies, reflecting an inhibition of hepatic fatty acid oxidation. Moreover, in rats treated with 4-methylpyrazole, the effect of the nucleoside on the increased rate of ethanol oxidation was not observed. The effect of adenosine on the modifications induced by the administration of ethanol in the mitochondrial redox potential was tested. When the nucleoside was administered to ethanol-treated animals, a reversal of the ethanol-induced diminution in the mitochondrial NAD^+/NADH ratio and in the redox potential was observed after 2–4 hr of treatment. These data lend further support to the suggestion that adenosine promotes the capacity of the cell to handle the reducing equivalents generated during ethanol metabolism. Moreover, these experiments suggest that hydrogen peroxide, generated through purine metabolism, plays a minimal role in the action of adenosine on ethanol metabolism. Finally, a second mechanism by which the nucleoside prevents fatty liver in the presence of allopurinol was evident and this was related to an inhibition of fatty acid metabolism.

It is well known that a great number of hepatic functions are altered after the ingestion of ethanol and that these effects are due mainly to the change in the redox state (NAD^+/NADH) produced by its metabolism. In addition, it has been suggested that reoxidation of cytosolic NADH is the rate-limiting step in ethanol metabolism [1–3].

It has also been found that administration of adenosine partially prevents ethanol-induced fatty liver, ameliorates the effect of ethanol metabolism on the cytoplasmic redox state, and increases ethanol oxidation [4]. Furthermore, ethanol enhances the adenosine-mediated increase in ATP [4, 5], and adenosine alone produces a marked shift in the mitochondrial redox state NAD^+/NADH [6]. Taking all these data into account, it has been suggested that the nucleoside may promote translocation of reducing power from the cytoplasm to the mitochondria, and that this mechanism may be responsible for the effects observed [4].

Hepatic cells catabolize adenosine to uric acid very rapidly [7], and in this pathway xanthine oxidase generates hydrogen peroxide. It has been shown that catalase is capable of oxidizing ethanol *in vitro* in the presence of a hydrogen peroxide-generating system [8]. Therefore, although it is generally accepted that, under basal conditions, hydrogen peroxide-mediated ethanol peroxidation does not play a significant role in ethanol metabolism [9, 10], it is theoretically possible that the effects of adenosine on ethanol metabolism might be due to this process.

This point was experimentally tested using allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine], a well-known inhibitor of xanthine oxidase, and 4-methylpyrazole, a potent inhibitor of alcohol dehydrogenase. The present paper shows that the action of adenosine on ethanol metabolism is mediated by alcohol dehydrogenase and the consequent alterations in the cytoplasmic and mitochondrial redox states. It is demonstrated that hydrogen peroxide, generated through purine catabolism, is not an important factor in adenosine action on ethanol-induced metabolic changes. Finally, data are presented showing the effect of the nucleoside on the metabolism of fatty acids originating from triglyceride stores, and the role of these fatty acids in the pathogenesis of ethanol-induced fatty liver.

MATERIALS AND METHODS

Adenosine, allopurinol, zeolite, alcohol dehydrogenase, α-glycerophosphate dehydrogenase, 3-hydroxybutyrate dehydrogenase and uricase were obtained from the Sigma Chemical Co. (St. Louis, MO). Coenzymes were purchased from Boehringer und Soehne (Mannheim, West Germany). 4-Methylpyrazole was a gift from Dr. A. I. Cederbaum. Other chemicals used were reagent grade of the best quality available.

The experiments were performed with male Wistar rats (weighing between 170 and 210 g) which were fasted for 16–20 hr. Ethanol was administered

through a stomach tube at a dose of 2.5 or 5 g/kg body wt. Control animals received an isocaloric dose of glucose. Immediately after gastric intubation, the animals were injected intraperitoneally with saline (NaCl, 0.85%) or adenosine suspended in saline at doses of 200 mg/kg body wt. Allopurinol (20 mg/kg body wt) or 4-methylpyrazole (200 mg/kg body wt) was suspended by homogenization either in saline or in the adenosine suspension. Thus, the animals were injected only once and were not subjected to any extra water or electrolyte load.

Hepatic triacylglycerols were determined by the method of Butler *et al.* [11]. Serum triacylglycerols and serum free fatty acids were assayed according to the methods of Van Handel and Zilversmit [12] and Dole and Meinertz [13], respectively. Blood and hepatic ketone bodies were quantified enzymatically: 3-hydroxybutyrate (3-OHB) according to Williamson and Mellamby [14], and acetoacetate (AA) according to Mellamby and Williamson [15]. Total ketone bodies were calculated as the sum of 3-OHB plus AA. Blood ethanol [16] and plasma uric acid [17] were estimated enzymatically. For the preparation of liver extracts, the animals were killed by a blow on the head. The abdomen was opened immediately with a bistoury and 150–300 mg of liver were homogenized in ice-cold 6% (w/v) perchloric acid within 7 sec after the abdominal incision. The homogenate was centrifuged at 300 *g* for 10 min, and the supernatant fraction was removed and carefully neutralized with 5 M K_2CO_3. Hepatic α-glycerophosphate (α-GP) and dihydroxyacetone phosphate (DHAP) were determined by the methods of Hohorst [18] and Bucher and Hohorst [19], respectively, and 3-hydroxybutyrate and acetoacetate as mentioned previously.

The activity of hepatic xanthine oxidase was quantified in a whole homogenate (20% in 0.25 M sucrose) according to Luck [20]. The activity is expressed as mK/mg protein, where K is the activity number of xanthine oxidase [20]. Protein was determined by the biuret method [21]. The cytoplasmic $NAD^+/NADH$ ratio was calculated using the equilibrium constant of α-glycerophosphate dehydrogenase as obtained by Russman [22] and taken from the paper by Veech *et al.* [23]. The mitochondrial redox state was calculated from the 3-OHB/AA ratio, using the equilibrium constant reported by Krebs [24] for the 3-hydroxybutyrate dehydrogenase. Redox potentials were calculated using the Nernst equation:

$$E_h = E_0' + 0.03 \log \frac{\text{electron acceptor}}{\text{electron donor}}$$

$$E_h = 0.314 + 0.03 \log \frac{NAD^+}{NADH}.$$

Statistical significance between comparable groups was determined by Student's *t*-test.

RESULTS AND DISCUSSION

The ability of allopurinol to inhibit xanthine oxidase was tested. Eight hr after administration, allopurinol produced about 30 per cent inhibition of the activity of this enzyme (Table 1). Surprisingly, adenosine or adenosine + ethanol magnified this effect, a 60 per cent inhibition (compared to the glucose + saline group) being observed in both cases (Table 1). Adenosine alone produced a small inhibition, while only minor changes in the activity of the enzyme were observed with ethanol (Table 1). The additive action of allopurinol and adenosine on xanthine oxidase may be due to a mutual competition for their metabolism. However, the *in vivo* inhibition of purine catabolism in the presence of allopurinol was 95 per cent, since a drop of this magnitude was observed in plasma uric acid levels (results not shown). In any event, the amount of hydrogen peroxide generated through the catabolism of purine was probably diminished to a minimum in rats treated with ethanol + adenosine + allopurinol.

Table 1. Effects of ethanol or glucose and adenosine or saline on the inhibition of xanthine oxidase by allopurinol*

	Activity of xanthine oxidase (mK/mg protein)	
Treatment	Without allopurinol	With allopurinol
Glucose + saline	246.57 ± 12.59 (4)	168.37 ± 26.01† (4)
Glucose + adenosine	203.59 ± 23.37 (3)	102.07 ± 5.39‡§ (4)
Ethanol + saline	229.20 ± 17.94 (4)	152.65 ± 19.28‖ (3)
Ethanol + adenosine	195.86 ± 6.50¶ (3)	99.61 ± 23.29** (4)

* Results are expressed as means ± S.E.M. with the number of animals in parentheses. Determinations were made 8 hr after treatment.

† $P < 0.05$, compared to the glucose + saline group (without allopurinol).

‡ $P < 0.01$, compared to the glucose + adenosine group (without allopurinol).

§ $P < 0.05$, compared to the glucose + saline group (with allopurinol).

‖ $P < 0.05$, compared to the ethanol + saline group (without allopurinol).

¶ $P < 0.02$, compared to the glucose + saline group (without allopurinol).

** $P < 0.02$ compared to the ethanol + adenosine group (without allopurinol).

The ability of adenosine to prevent the ethanol-induced fatty liver was tested in the presence and absence of allopurinol. Eight hr after ethanol administration a 4-fold accumulation of triacylglycerol in the liver was observed, which was partially reversed by adenosine (48 per cent diminution), as reported previously [4]. These effects were essentially unaltered by xanthine oxidase inhibition, but when allopurinol and adenosine were administered together, no ethanol-induced accumulation of lipids was detected. A value of 2.87 ± 0.49 mg hepatic triacylglycerols/g wet wt was detected in the group given glucose and saline, which is similar to that obtained in the rats subjected to ethanol + adenosine treatment (2.15 ± 0.45 mg triacylglycerols/g wet wt) (figures are expressed as the mean ± S.E. of six determinations in each group). Thus, a total prevention of ethanol-induced fatty liver, in the presence of adenosine and allopurinol, was demonstrated.

To study ethanol oxidation, a small dose of ethanol (1 g/kg) was given to rats treated with allopurinol or allopurinol + adenosine, the animals being killed 4 hr after treatment. Under these conditions, the blood level of ethanol was 9.44 ± 1.44 mM in rats treated with allopurinol and 4.92 ± 0.26 mM in rats treated with allopurinol + adenosine (mean ± S.E.M. of five determinations in each case, $P < 0.02$). To further test if there was an increase in ethanol oxidation, 4-methylpyrazole, a specific inhibitor of alcohol dehydrogenase, was employed. Ethanol (2.5 g/kg body weight) was administered to rats treated with pyrazole or pyrazole + adenosine, and the levels of ethanol in the blood were subsequently measured. No significant difference was detected between the groups (results not shown), suggesting that adenosine treatment increased the flux of ethanol *in vivo* through alcohol dehydrogenase.

A clear correlation between liver triacylglycerol content and cytoplasmic redox state was presented in a previous paper [4]. Therefore, the cytoplasmic redox state calculated from the levels of a cytoplasmic pair α-GP and DHAP, was studied in rats treated with allopurinol. The administration of allopurinol did not affect significantly the cytoplasmic redox state in the presence of ethanol with or without adenosine. As expected, ethanol produced a large accumulation of α-glycerophosphate, reflecting a drastic decrease in the cytoplasmic $NAD^+/NADH$ ratio. Adenosine significantly ameliorated this effect of ethanol metabolism by a magnitude similar to that observed previously in the absence of allopurinol [4]. The strong correlation previously observed between the cytoplasmic redox state and the amount of triacylglycerols in the liver [4] was not observed in rats treated with allopurinol, the correlation coefficient being 0.56. This is probably due to the fact that adenosine completely prevented the accumulation of triacylglycerols produced by ethanol and partially modified the effect of the alcohol on the redox state, increasing by 30–40 per cent the $NAD^+/NADH$ over the control group. Hence, in addition to its effect on the cytoplasmic redox state, adenosine probably prevents the ethanol-induced fatty liver by a second mechanism, as evidenced in the presence of allopurinol.

In an effort to clarify this second mechanism manifested by adenosine, the concentration of serum lipids and the blood level of ketone bodies, as an index of the oxidation of fatty acids by the liver [25], were investigated. Allopurinol did not modify to an appreciable extent the effect of adenosine alone, or in the presence of ethanol, on the level of triacylglycerols in serum. As reported previously, ethanol produced an increase in serum triacylglycerols, and the administration of adenosine did not increase the triacylglycerolemia but decreased it to normal levels. It is interesting that in the four experimental groups allopurinol treatment decreased triacylglycerolemia by about 20 per cent.

Adenosine is a powerful antilipolytic agent [26] and, although this action does not seem to play an important role in its effect on fatty liver [4], the possibility of a magnification by allopurinol of the antilipolytic action of adenosine was considered. Contrary to what was expected, in the presence of allopurinol, which inhibits purine catabolism, adenosine increased serum-free fatty acid levels, the concentrations detected being 41.5 ± 7.6 μ-equiv./100 ml of serum in the glucose + saline group and 56.6 ± 4.7 μ-equiv./100 ml in the presence of adenosine. This action was more evident in animals treated with ethanol, where a marked increase in serum-free fatty acids was observed (54.3 ± 2.3 in the ethanol + saline group and 68.8 ± 43.3 in the ethanol + adenosine-treated animals; mean ± S.E. of samples taken from six animals in each experimental group after 8 hr of the treatment, $P < 0.02$).

The level of free fatty acids in the serum of rats is the result of a balance between lipolysis, mainly in adipose tissue, and utilization, mainly by the liver. Savolainen *et al.* [27] have shown that *in vivo* ethanol administration increases adipose tissue cyclic AMP. However, no significant increases in serum-free fatty acids were observed by these authors [27] or by us. This is probably due to the increased hepatic uptake of free acids from serum in ethanol-treated rats [28]. The increased serum level of fatty acids observed in rats treated with ethanol + adenosine + allopurinol probably resulted from an enhanced lipolysis produced by ethanol and a decreased free fatty acid uptake by the liver due to adenosine [29]. On the other hand, the action of adenosine in inhibiting hepatic fatty acid oxidation that results in decreased plasma ketone body levels [28] was observed in the presence of allopurinol. This action of the nucleoside was more evident after 4 hr of treatment, as evidenced by an inhibition of the ketonemia of 77 per cent in the animals receiving a glucose load and 54 per cent in those treated with ethanol. Ethanol increased the amount of 3-OHB and the total amount of ketone bodies, and both actions were blocked by adenosine. The results previously obtained [30] in the absence of allopurinol persisted for 1 hr, but in the presence of this drug the effect was optimal after 4 hr of treatment, and a 20 per cent inhibition of ketonemia was still evident after 8 hr. As yet, we have no explanation for the observed effects of allopurinol in prolonging the action of adenosine. However, under these conditions, fatty acids were neither esterified nor oxidized, i.e. no accumulation of triacylglycerols in the liver was detected, the

hyperlipemia produced by ethanol was not increased but rather decreased to normal levels, and the concentration of ketone bodies was not augmented but decreased. Therefore, a reasonable explanation of these observations is that these fatty acids are not metabolized by the liver under these conditions. This point is supported by the fact that adenosine inhibits the extramitochondrial activation of fatty acids [30] which is essential for their hepatic metabolism.

The effects of adenosine alone on the mitochondrial redox state have already been published and discussed [6] and are consistent with the hypothesis that the nucleoside enhances the utilization of reducing power by the respiratory chain. The results obtained for these variables in the presence of ethanol and/or adenosine and their controls, receiving a glucose load, are presented in Table 2. One hour after treatment with glucose the animals exhibited a low rate of fatty acid oxidation. If ketogenesis is considered an index of hepatic fatty acid oxidation (Table 2), this probably occurs because, following glucose administration, the liver should have a low metabolic rate, since glucose is available for consumption by extrahepatic tissues, and it is not necessary for the liver to produce ketone bodies or glucose. However, the mitochondrial redox state, expressed as $NAD^+/NADH$, was kept at the normal value reported previously [6]. After 4 hr of treatment, a decrease in the mitochondrial $NAD^+/NADH$ ratio in the control animals receiving a glucose load was observed, probably due to hormonal adjustment, since the glucose absorption must be terminated after the second hour. Adenosine action was observed in these conditions, and 1 hr after the treatment of glucose and adenosine a marked decrease in 3-OHB (reduced substrate) was observed. Consequently, the $NAD^+/NADH$ ratio increased, which is in agreement with data reported previously without the glucose load [6].

The metabolism of ethanol produces acetaldehyde which is oxidized within the mitochondria by acetaldehyde dehydrogenase, generating NADH and, so, exceeding the capacity of utilization by the respiratory chain thereby modifying the mitochondrial redox state. As expected, ethanol produced a marked decrease in the mitochondrial $NAD^+/NADH$ ratio at all times tested, due mainly to an appreciable increase of 3-OHB (reduced substrate).

The administration of adenosine produced an increase in the metabolism of ethanol [4], thus increasing the supply of acetaldehyde to the mitochondria and, consequently, leading to a further excess of NADH which will result in a decrease of the $NAD^+/NADH$ ratio more marked than with ethanol alone. The action of adenosine, however, was exactly the opposite, i.e. the nucleoside increased the mitochondrial redox state (Table 2) by decreasing the level of 3-OHB (reduced substrate) until 4 hr of the treatment. No significant changes in the levels of acetoacetate were observed after nucleoside treatment. The action of adenosine on these variables was not evident after 8 hr of treatment. These data lend further support to the suggestion that reducing equivalents generated by the oxidation of ethanol are utilized faster by the respiratory chain. This could be a consequence of an increased transfer of reducing power from the cyto-

Table 2. Effects of ethanol or glucose and adenosine or saline on the hepatic mitochondrial redox state

Time (hr)	Treatment	3-OHB (μmoles/g wet wt)	AA (μmoles/g wet wt)	Total ketone bodies (3-OHB + AA) (μmoles/g wet wt)	$NAD^+/NADH$
1	Glucose + saline	163.7 ± 21.3	196.4 ± 36.8	360.2	24.44
1	Glucose + adenosine	48.8 ± 7.0†	196.6 ± 27.5	245.4	81.63
1	Ethanol + saline	585.7 ± 87.7†	256.6 ± 38.9	842.3	8.90
1	Ethanol + adenosine	501.1 ± 59.8†	229.6 ± 44.1	730.7	9.29
2	Glucose + saline	136.7 ± 18.7	149.6 ± 43.5	286.4	22.29
2	Glucose + adenosine	129.7 ± 22.5	203.3 ± 27.4	333.0	31.69
2	Ethanol + saline	531.3 ± 50.9‡	175.90 ± 43.53	707.2	6.72
2	Ethanol + adenosine	351.9 ± 29.2§‖	171.6 ± 32.3	523.5	9.89
4	Glucose + saline	346.0 ± 44.5	211.7 ± 25.5	557.7	12.44
4	Glucose + adenosine	198.0 ± 30.1¶	225.6 ± 46.9	423.6	23.05
4	Ethanol + saline	886.2 ± 113.7**	325.8 ± 46.3	1212.0	7.46
4	Ethanol + adenosine	599.0 ± 100.7††	325.5 ± 53.2	924.5	11.02
8	Glucose + saline	291.2 ± 24.1	240.1 ± 42.4	531.3	16.76
8	Glucose + adenosine	288.1 ± 40.9	233.3 ± 34.6	521.3	16.49
8	Ethanol + saline	568.5 ± 101.8‡‡	223.0 ± 38.0	791.4	7.95
8	Ethanol + adenosine	671.1 ± 117.52§§	211.17 ± 23.82	882.3	6.38

* Results are expressed as the mean ± S.E.M. of at least four animals for each group.
† $P < 0.005$, compared to the glucose + saline group (1 hr).
‡ $P < 0.001$, compared to the glucose + saline group (2 hr).
§ $P < 0.001$, compared to the glucose + saline group (2 hr).
‖ $P < 0.02$, compared to the ethanol + saline group (2 hr).
¶ $P < 0.025$, compared to the glucose + saline group (4 hr).
** $P < 0.005$, compared to the glucose + saline group (4 hr).
†† $P < 0.05$, compared to the glucose + saline group (4 hr).
‡‡ $P < 0.025$, compared to the glucose + saline group (8 hr).
§§ $P < 0.01$, compared to the glucose + saline group (8 hr).

plasm to the mitochondria, an increase in the flux of the respiratory chain, or by a synergy of the two mechanisms. Although this point will not be discussed here, in Table 3 we present the mitochondrial redox potential calculated from Table 2, the cytoplasmic redox potentials calculated from data in our previous paper [4], and the differences between both compartments. Adenosine alone produced an increase of 15 mV in the mitochondrial redox potential 1 hr after its administration. This action diminished to 4 and 8 mV after 2 and 4 hr, respectively, and then disappeared. No effect of adenosine alone on the cytoplasmic redox potential was detected (Table 3). Ethanol decreased (became more negative) the redox potential in both compartments. In relation to its effects on ethanol metabolism, the nucleoside produced an elevation of 5 mV in the mitochondrial compartment and 8–6 mV in the cytoplasm after 1, 2 and 4 hr of the treatment. Eight hr after treatment the action of adenosine on the cytoplasm was still apparent, although no effect was detected within the mitochondria. It is interesting, however, that adenosine only modifies the cytoplasmic redox state when there is a low NAD^+/NADH ratio, i.e. during ethanol oxidation. The differences in redox potential between the two compartments remained fairly constant during the course of the experiment (Table 3), regardless of the treatment and of the cytoplasmic and mitochondrial modifications induced by ethanol alone or with adenosine. Although at the moment it is difficult to understand the physiological meaning of the requirement to keep at around 50 mV the difference between cytoplasmic and mitochondrial redox potential, it is evident that the constancy of this equilibrium was maintained. No evidence for a direct interaction of adenosine and ethanol metabolism is presented, but it seems to involve an indirect action mediated through fatty acid and mitochondrial metabolisms. The inhibition of fatty acid oxidation by ethanol has been reported [31], the effect being measurable after 1 or 2 hr of the treatment (Table 2) and potentiated by the presence of adenosine. It is important to emphasize that the inhibition of fatty acid oxidation induced by ethanol is accompanied by an increase in triglyceride biosynthesis, whereas adenosine inhibition is concomitant with an inhibition in triglyceride formation.

In summary, two factors seem to be involved in the pathogenesis of ethanol-induced fatty liver: an increased supply of fatty acids to the liver concomitant with an enhanced uptake and an increased availability of α-GP. Adenosine action on ethanol-induced fatty liver does not appear to be related to an H_2O_2-mediated ethanol peroxidation but to its effect on the cytoplasmic redox state limiting the availability of α-GP, and to an enhanced translocation and oxidation of the reducing power.

In the presence of allopurinol, another mechanism of adenosine-mediated prevention of ethanol-induced fatty liver became evident, an inhibition of fatty acid metabolism. Therefore, adenosine prevents fatty liver by limiting the availability of the precursors for triacylglycerol synthesis, α-GP and acylCoA. At the moment, however, it is difficult to correlate both actions of adenosine that prevent the ethanol-induced fatty liver.

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Table 3. Comparative action of ethanol or glucose and adenosine or saline on the cytoplasmic and mitochondrial redox potentials*

Time (hr)	Treatment	$E_{Cytoplasmic}$ (mV)	$E_{Mitochondrial}$ (mV)	$E_{(C-M)}$ (mV)
1	Glucose + saline		−272.36	
1	Glucose + adenosine		−256.64	
1	Ethanol + saline		−285.52	
1	Ethanol + adenosine		−284.96	
2	Glucose + saline	−217.88	−273.56	55.68
2	Glucose + adenosine	−217.13	−268.97	51.84
2	Ethanol + saline	−236.26	−289.18	52.92
2	Ethanol + adenosine	−228.05	−284.14	56.09
4	Glucose + saline	−219.89	−281.16	61.27
4	Glucose + adenosine	−222.18	−273.12	50.94
4	Ethanol + saline	−237.12	−287.82	50.70
4	Ethanol + adenosine	−230.76	−282.73	51.97
8	Glucose + saline	−225.84	−277.27	51.43
8	Glucose + adenosine	−225.90	−277.48	51.58
8	Ethanol + saline	−246.73	−286.99	40.26
8	Ethanol + adenosine	−237.74	−289.86	52.12

* Results were calculated from data taken from Table 1 and Ref. 1.

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